

Original Research Article

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Testicular Volume, Seminal Attributes and Body condition score in Infertile Dogs

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ABSTRACT

Keywords

Aspermia, Azoospermia, Hypokinozoospermia, Oligozoospermia, Poor membrane integrity, Semen collection, Testicular degeneration

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The six different breeds of dogs were selected for Breeding Soundness Examination. The testes were soft in texture and no other scrotal abnormality was noticed. Body condition score was evaluated on a scale of 1-9 and Testicular volume was measured by Vernier caliper before semen collection. Semen was collected by digital manipulation. The biophysical and biochemical characteristics of semen were analyzed. The body condition score, testicular volume, volume of semen, colour, pH, mass activity, initial motility, sperm concentration, live spermatozoa, total sperm abnormality, Intact acrosome and positive for HOS were 4.80 ± 0.32 , $23.4 \pm 1.94 \text{ cm}^3$, $3.90 \pm 0.42 \text{ ml}$, Opalescent/clear, 6.12 ± 0.06 , 2.58 ± 0.11 , $54.8 \pm 4.39\%$, $110 \pm 18.52 \text{ millions/ml}$, $70.2 \pm 2.60\%$, $11.1 \pm 2.06\%$, $38.3 \pm 4.58\%$ and $35.2 \pm 4.67\%$, respectively. The total protein, albumin, globulin, ALP and ACP were $2.47 \pm 0.29 \text{ g/dl}$, $0.28 \pm 0.05 \text{ g/dl}$, $1.96 \pm 0.24 \text{ g/dl}$, $1904 \pm 329 \text{ IU/L}$ and $925 \pm 79.0 \text{ IU/L}$, respectively. The major poor semen quality parameters in this study were oligozoospermia, hypokinozoospermia, azoospermia, aspermia, testicular degeneration, poor membrane integrity, poor secretory functions of the epididymis and prostate gland. In the present study all the nine dogs were infertile.

Introduction

A rapid, reliable and cost effective treatment to predict ovulation time by vaginal cytology is widely practiced to fix breeding date. In spite of fixing breeding date by different methods like Exfoliative Vaginal Cytology, Blood progesterone estimation, Vaginoscopy, a wide spread apparent infertility in dogs has been reported by pet owners and dog breeders. Reduced fertility and fecundity in dog results in substantial financial losses to the canine breeding industry. In this context recently canine semen has been attracting attention by

researches. The key aspects of Breeding Soundness Evaluation are body condition score, testicular volume and semen evaluation. Body Condition Score was used to assess the general health of dogs on a scale of 1-9, where 1 refers to emaciated animal and 9 indicates grossly obese dog (Edney and Smith, 1986). Testicular biometry was calculated by measuring testicular length and width by using verniercaliper (Harriet *et al.*, 2002). The most commonly used method for semen collection in dogs is by digital manipulation (Linde-Forsberg, 2005a). Semen parameters such as semen volume, sperm concentration, motility,

live and dead spermatozoa and sperm morphology in the whole ejaculate are frequently examined to assess the quality of semen (Silva *et al.*, 2005). The functional integrity of the sperm plasma membrane is crucial for the viability and fertilizing ability of spermatozoa (Hafez, 1993). The determination of intact acrosome is another essential tool to evaluate indirectly the fertilizing capacity of spermatozoa (Wells and Awa, 1970). The seminal plasma enzyme, alkaline phosphatase is primarily of testicular and epididymal origin. Alkaline phosphatase level is often used for the diagnosis of oligozoospermia, azoospermia and incomplete ejaculation (Schafer-Somi *et al.*, 2013). Another seminal plasma enzyme, the acid phosphatase is a biochemical marker, primarily related to the metabolic functions of spermatozoa and prostate diseases in dogs (King and Macpherson, 1966). The objective of this study, all the different breeds of dogs was screened for BSE and all the semen quality measures comparable to other reports on the fertile dog semen.

Materials and Methods

Experimental animals

The six different breeds of dogs aged between 2-6 years maintained by dog breeders and pet owners of Puducherry were selected for Breeding Soundness Examination. The six different breeds of dogs comprised of GSD, Labrador, Pug, Cocker spaniel, Golden Retriever and Spitz. All the dogs were apparently healthy and they were fed twice a day with commercial dry dog food and had *ad libitum* access to water.

Body condition score

Body Condition Score (BCS) on a scale of 1 to 9 was assessed before semen collection as per the method described by Laflamme

(1997). The mid-range in the scale represented optimal body condition, lower values indicated lean to emaciated conditions and higher value to excessive body fat.

Testicular volume

The measurement of testicular dimensions (length, width and height) provides an accurate assessment of canine testicular volume. The testicular volume was measured as per Vernier caliper method and as per the formula adopted by Harriet *et al.*, (2002).

Semen collection

Semen was collected by digital manipulation and stimulation of the *Bulbus glandis* (Linde-Forsberg, 2005a). The whole ejaculates (including pre-sperm, sperm rich and post sperm) were collected in sterile graduated measuring plastic tube. Immediately the collected semen was brought to the laboratory in a transport container. Sufficient care was taken to ensure that the semen was not exposed to unfavourable conditions during and after collection. The first semen collection was used as test trial and not included in the analysis of data and subsequently the semen was collected at weekly interval for six consecutive weeks and subjected to semen evaluation. A total of 37 ejaculates from nine dogs were subjected to semen analysis. During the study period the selected dogs was not subjected to additional semen collection or natural service.

Volume and colour of the ejaculate

A glass funnel fitted in a graduated measuring plastic tube was used to collect the ejaculate and the volume was directly measured on the graduated marks in the measuring tube (Linde-Forsberg, 1995). The colour was visually noticed as per the method described by (Feldman *et al.*, 2004).

pH and mass activity

The pH of dog semen was measured by pH paper in the pH range from 5.0-8.0 (Freshman, 2002). The mass activity of spermatozoa was recorded immediately after semen collection by examining a drop of semen on a clean glass slide at 100x magnification without cover slip. Mass activity was evaluated on a scale of 0-5 as per the method described by Evans and Maxwell (1987).

Initial motility

The initial motility of spermatozoa was recorded immediately after semen collection by examining a drop of semen on a clean glass slide and diluted with one drop of Tris buffer kept at 37°C at 400x magnification with a coverslip. The initial motility was evaluated as per the method described by Gunzel-Apel (1994) and Feldman and Nelson (1996).

Sperm concentration

Sperm concentration was calculated by haemocytometric method (Sorenson, 1976) and expressed in millions of spermatozoa per milliliter of semen. A small volume of semen (20 microliters) was added to a known volume of formalin based fluid to kill the sperm and a known volume of this fluid is applied to a counting chamber in the haemocytometer. The counting chamber's grid under a microscope at 400x magnification, the numbers of spermatozoa were counted to determine the concentration per milliliter of semen.

Live and Dead spermatozoa

Nigrosin-eosin staining technique (Hancock and Rowlands, 1949) was adopted to identify the live and the dead spermatozoa. A total of 200 spermatozoa counted in randomly selected microscopic fields and the percentage of live spermatozoa was calculated.

Sperm abnormality

The sperm morphology was studied using nigrosin-eosin stained semen smear. A total of 200 sperms will be observed in not less than 10 randomly selected microscopic fields per sample under 1000x magnification.

The percentage of abnormal sperm with head abnormality, proximal and distal protoplasmic droplet, deformed tail and total number of abnormal sperm were calculated.

Membrane integrity

Hypo-Osmotic Swelling Test (HOST)

The sperm plasma membrane integrity was assessed as per the method described by Lomeo and Giambersio (1991). A volume of 10.0 µl of semen was added to 1 ml of mono distilled water and incubated at 37°C for 5 min. After incubation, 1 drop of the semen was placed on a glass slide covered with cover slip and evaluated under 1000x magnification. A total of 200 sperms were evaluated and percentages of swollen tail spermatozoa were calculated.

Acrosome membrane integrity

Acrosomal cap integrity by using Giemsa stain was studied as per the method adopted by Watson (1975). The acrosome morphology was classified as intact (Plate 5), partial and completely lost acrosome as per the classification of Blom (1972).

Biochemical constituents in canine seminal plasma

The semen collected was centrifuged at 1000x g for 15 min at room temperature. The separated seminal plasma was centrifuged at 10,000x g for 10 min at room temperature and stored at -80°C (Strzezek *et al.*, 2015). The separated seminal plasma was used for

estimation of biochemical constituents.

Estimation of total protein, albumin and globulin

The total protein and albumin in the seminal plasma was estimated by colorimetric method (Doumas *et al.*, 1977). The diagnostic kits of Beacon diagnostic was used for estimating the concentration of total protein and albumin expressed in mg/ml by using a A_{546} nm for total protein and A_{630} nm for total albumin. The total globulin (G) concentration was calculated based on the difference between seminal plasma total protein and seminal plasma albumin.

Estimation of alkaline and acid phosphatase

The concentration of alkaline phosphatase (Bessey *et al.*, 1946) and acid phosphatase enzymes were estimated by colorimetric method A_{405} nm by using the diagnostic kits.

Statistical analysis

The data generated through semen evaluation in different breeds of dogs was analyzed statistically by One-way ANOVA followed by turkey test by using the Graph pad prism version 5.

Results and Discussion

The nine infertile dogs comprised of GSD (n:1), Labrador (n:2), Pug (n:3), Cocker spaniel (n:1), Golden Retriever (n:1) and Spitz (n:1). The mean \pm SE for age was 3.55 ± 0.50 . A total of 37 ejaculates were analyzed and compared with fertile dog semen. For infertile dogs, the data generated for BCS, testicular volume, biophysical and biochemical constituents of semen were analyzed and compared with fertile dogs.

Breeding soundness examination

Biophysical characteristics of semen

The mean body condition score for infertile dogs was 4.80 ± 0.32 . The testicular volume was 23.4 ± 1.94 cm³ (Fig. 1), volume of the ejaculate 3.90 ± 0.42 ml and the pH of semen was 6.12 ± 0.06 for infertile dogs. The mass activity (2.58 ± 0.11), initial motility ($54.8 \pm 4.39\%$), sperm concentration (110 ± 18.52 millions /ml) and the incidence of percentage of live spermatozoa ($70.2 \pm 2.60\%$) were low in infertile compared to fertile dog semen. The total sperm abnormality was $11.1 \pm 2.06\%$ in infertile dogs. The mass activity (2.58 ± 0.11), initial motility ($54.8 \pm 4.39\%$), sperm concentration (110 ± 18.52 millions/ml) and the incidence of percentage of live spermatozoa ($70.2 \pm 2.60\%$) were low in different breeds of dogs comparable with other reports on fertile dog semen. The total sperm abnormality was $11.1 \pm 2.06\%$ in dogs (Table 1).

Sperm membrane integrity

Acrosome membrane integrity

The percentage of incidence of intact acrosome was $38.3 \pm 4.58\%$ and the HOS positive spermatozoa were $35.2 \pm 4.67\%$ for dogs. The acrosome integrity was classified as intact acrosome, partially lost acrosome and completely lost acrosome were 38.3 ± 4.58 Vs $79.2 \pm 0.63\%$, 23.9 ± 3.48 Vs $13.6 \pm 0.62\%$ and 37.7 ± 4.47 Vs $7.47 \pm 0.49\%$, respectively (Table 1). The percentage of intact acrosome was significantly low in the present study compared to other reports on fertile dog semen.

Hypo Osmotic Swelling Test (HOST)

Spermatozoal response to HOST were classified three types, strongly coiled,

moderately coiled and tip coiled and their percentage of incidence were $10.3 \pm 0.97\%$ Vs 35.46 ± 0.68 , 11.6 ± 1.38 Vs $24.26 \pm 1.21\%$ and 42.7 ± 2.33 Vs $15.5 \pm 0.99\%$, respectively (Table 1). The strongly coiled spermatozoa were very low in the present study compared to fertile dogs. On the contrary, the tip coiled spermatozoa were high in the present study.

Biochemical characteristics of semen

The Total protein, albumin and globulin level in the seminal plasma of different breeds of dogs were 2.47 ± 0.29 g/dl, 0.28 ± 0.05 g/dl and 1.96 ± 0.24 g/dl, respectively. The alkaline and acid phosphatase level in the seminal plasma was 1904 ± 329 IU/L and 925 ± 79.0 IU/L, respectively in breeds of dogs (Table 2). The ALP and ACP level in the seminal plasma were low in the present study compare to other reports on fertile dog semen.

Based on Breeding Soundness Examination, the biophysical and biochemical characteristics of semen were significantly low in the present study. Among the nine dogs subjected to BSE, all the dogs were infertile.

Among the infertile dogs the incidence of detached sperm head and distal cytoplasmic droplets were predominant (Fig. 2 and 3).

The percentage of incidence of dead spermatozoa, HOS negative, acrosome defects were high for infertile dogs. In both infertile dogs the incidences of abnormal spermatozoa were within permissible level, but however detached sperm head and distal cytoplasmic droplets were predominant in infertile dogs (Fig. 2 and 3).

The various poor semen quality parameters among infertile dogs observed and identified in this study were Aspermia (Fig. 4), Azoospermia, Oligo and hypokinozoospermia and poor membrane integrity of spermatozoa.

The epididymal secretary functions are largely reflected by ALP level in the seminal plasma and they were very low in all infertile dogs compared to fertile dog's semen.

Infertile dogs comprised of nine dogs (GSD; n=1, Labrador; n=2, pug; n=3, Golden Retriever; n=1, Cocker spaniel; n=1 and Spitz; n=1). In infertile dogs, the testes were soft in texture and no other scrotal abnormality was noticed.

The semen was collected at weekly intervals for six consecutive weeks but Aspermia constitute 32% and the remaining ejaculate were analyzed and compared.

Fig.1 Measurement of testicular volume



Fig.2 Detached sperm head; Eosin-nigrosin stain (1000x magnification)

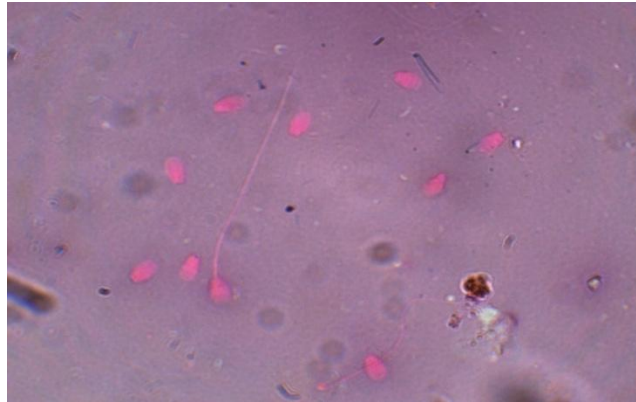


Fig.3 Distal cytoplasmic droplet; Eosin-nigrosin stain (1000x magnification)



Fig.4 Percentage of incidence of Aspermia in different breeds of infertile dogs

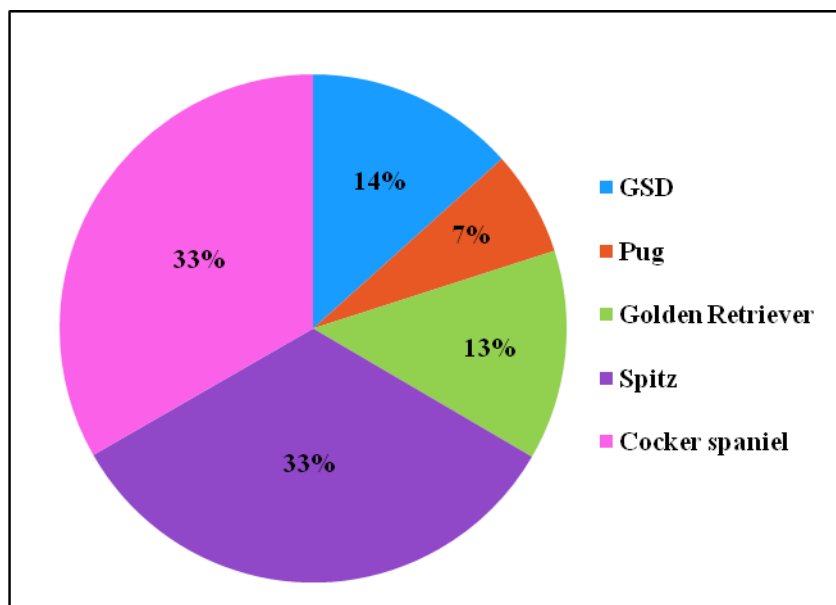


Table.1 Semen characteristics in different breeds of dogs

SI. No	Parameters	Breeds in dog					
		GSD	Labrador	Pug	Golden Retriever	Spitz	Cocker spaniel
1	BCS	3.00 ± 0.00	6.00 ± 0.30 ^b	4.33 ± 0.45 ^c	3	3	2
2	Testicular volume (cm ³)	24.5 ± 0.00 ^a	35.2 ± 1.71 ^b	13.7 ± 0.32 ^c	14.05	26.4	6.14
3	Volume of the semen (ml)	5.00 ± 0.57 ^a	5.95 ± 0.57 ^{ac}	1.97 ± 0.18 ^b	1.92±0.07	4.50	3
4	Colour of the semen	Clear	Opalescent	Clear/Opalescent	Clear	Clear	Clear
5	pH of the semen	5.50 ± 0.00 ^a	6.25 ± 0.07 ^{bc}	6.20 ± 0.06 ^c	5.75±0.14	6.50	5.50
6	Mass Activity of spermatozoa	2.00 ± 0.00 ^a	3.00 ± 0.00 ^b	2.40 ± 0.19 ^{ac}	Azoospermia	3	Azoospermia
7	Initial Motility of spermatozoa (%)	20.0 ± 0.00 ^a	78.3 ± 1.12 ^b	45.3 ± 4.86 ^c	Azoospermia	80	Azoospermia
8	Sperm Concentration (millions/ml)	20.0 ± 0.00 ^{ac}	210 ± 22.0 ^b	55.2 ± 16.2 ^c	Azoospermia	328	Azoospermia
9	Live Spermatozoa (%)	57.7 ± 2.49 ^{ac}	83.1 ± 2.27 ^b	63.2 ± 3.20 ^c	Azoospermia	93	Azoospermia
10	Total sperm Abnormality (%)	2.00 ± 0.40	8.29 ± 1.94	15.8 ± 3.58	Azoospermia	90	Azoospermia
11	Intact Acrosome (%)	8.70 ± 0.85 ^{ac}	58.6 ± 4.32 ^b	30.0 ± 6.00 ^c	Azoospermia	72	Azoospermia
12	HOS (+) (%)	3.00 ± 0.40 ^{ac}	53.4 ± 4.76 ^b	29.3 ± 6.43 ^c	Azoospermia	70	Azoospermia

Mean value having different super script within row differ significantly (P<0.05).

GSD - Two collections are Aspermia.

Pug - Three collections are Aspermia.

Golden Retriever - Two collections are Aspermia.

Spitz - Five collections are Aspermia.

Cocker spaniel - Five collections are Aspermia.

Table.2 Seminal plasma biochemical parameters in different breeds of dogs

SI. No	Parameters	Breeds in dogs					
		GSD	Labrador	Pug	Golden Retriever	Spitz	Cocker spaniel
1	Total Protein (g/dl)	1.52 ± 0.24 ^{ac}	3.66 ± 0.53 ^b	1.72 ± 0.26 ^c	1.47 ± 0.29	3.10	1.90
2	Albumin (g/dl)	0.17 ± 0.02	0.38 ± 0.12	0.24 ± 0.04	0.32 ± 0.13	1.40	0.90
3	Globulin (g/dl)	0.85 ± 0.06 ^{ac}	2.86 ± 0.43 ^b	1.54 ± 0.25 ^c	1.15 ± 0.25	1.70	1
4	ALP (IU/L)	1544 ± 587.7	2568 ± 185.3	1468 ± 631.5	172.8 ± 109.6	1806	2322
5	ACP (IU/L)	1341 ± 37.81	935.7 ± 120.3	807.5 ± 119.3	25.00 ± 6.45	213	230.0

Mean value having different super script within row differ significantly (P<0.05).

The mean body condition score for all infertile dogs was 4.80 ± 0.32 . The mean testicular volume, volume of the ejaculate and pH of semen (23.4 ± 1.94 , 3.90 ± 0.42 ml and 6.12 ± 0.06) were low in infertile dogs compared to fertile dogs. The BCS and testicular volume did not differ between weeks of evaluation in infertile dogs. Costa *et al.*, (2010) and Singh *et al.*, (2014) reported that as the body weight increased, the scrotal circumference also increased without parallel increase in testicular volume and sperm concentration.

In infertile dogs, seven out of nine infertile dogs gave clear and colourless ejaculate consistently. The remaining two dog ejaculates were opalescent throughout the study period and confirmed as normospermia. The dogs that gave clear and colourless ejaculates were confirmed as oligozoospermia in four dogs, azoospermia in one dog and the remaining two dogs were Aspermia.

The mean sperm concentration for all infertile dogs was 110.0 ± 18.52 millions / ml. The sperm concentration was low in infertile dogs compared to fertile dog semen. The testicular volume (23.4 ± 1.94 cm³) and sperm concentration (110.0 ± 18.52 millions / ml) were low in infertile dogs compared to fertile dogs. The sperm concentration in the ejaculate depended on testicular size and extragonadal sperm reserve (Amann, 1981 and Olar *et al.*, 1983).

In all oligozoospermic dogs, the spermatozoa showed hypokinozoospermia which was different from that of fertile dogs ($54.8 \pm 4.39\%$ initial motility and 2.58 ± 0.11 mass activity).

The alkaline phosphatase level in the seminal plasma was also less in infertile dogs. The ALP in the seminal plasma is a biochemical marker for epididymal function (Strzezek *et*

al., 2015). Hence in this study, in normospermic dogs the epididymal dysfunction could be the cause for infertility.

Among the infertile dogs the incidence of detached sperm head and distal cytoplasmic droplets were predominant whereas, tail abnormalities were common. The higher incidence of detached sperm head and distal cytoplasmic droplet indicated possible testicular and epididymal dysfunction. Ortega-Pacheco *et al.*, (2006b) reported that detached heads are common sperm abnormalities in canine testicular degeneration. Barth and Oko (1989) recorded that cytoplasmic droplets indicate pathological conditions of the epididymis.

The percentage of incidence of intact acrosome and HOS positive sperms were low in infertile dogs compared to fertile dogs. The percentage of intact acrosome was $38.3 \pm 4.58\%$ and HOS positive spermatozoa were $35.2 \pm 4.67\%$ for infertile dogs. The acrosome integrity was classified as intact acrosome ($38.3 \pm 4.58\%$), partially lost acrosome ($23.9 \pm 3.48\%$) and completely lost acrosome ($37.7 \pm 4.47\%$). Based on the type of sperm swelling the HOS positive sperms were classified as strongly coiled ($10.3 \pm 0.97\%$), moderately coiled ($11.6 \pm 1.38\%$) and tip coiled ($42.7 \pm 2.33\%$). The strongly coiled tail was low in infertile dogs compared to fertile dogs. On the contrary the incidence of tip coiling was high in infertile dogs. Hence from the study it can be conclude that there was poor in the permeability of plasma membrane and acrosome integrity in infertile dogs.

In infertile dogs the total protein, albumin and globulin were 2.47 ± 0.29 g/dl, 0.28 ± 0.05 g/dl and 1.96 ± 0.24 g/dl, respectively and the values were not different from fertile dogs. The ALP and ACP level in the seminal plasma were low in infertile dogs compared to

fertile dogs. The ALP and ACP levels were 1904 ± 329 Vs 925 ± 79.0 in infertile. Hence, the low level of ALP and ACP could be due to secretory dysfunction of epididymis and prostate gland, since both ALP and ACP were the biochemical markers for the functional integrity of epididymis and prostate gland respectively.

On enquiry from dog owners all the infertile dogs had mated previously during two breeding seasons with no resultant pregnancy. Infertile dogs were systematically screened for BSE and all the semen quality measures comparable to other reports on the fertile dog semen (Gunay *et al.*, 2003; Kurien *et al.*, 2012; Domoslawska *et al.*, 2013). The various poor semen quality parameters among infertile dogs observed and identified in this study were Aspermia, Azoospermia, Oligo and hypokinozoospermia and poor membrane integrity of spermatozoa. The epididymal and prostate secretory functions are largely reflected by ALP and ACP level in the seminal plasma and they were very low compared to fertile dog semen. Hence it can be concluded that the epididymal and prostate secretory functions were also poor.

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